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(54) USE OF 2,5-DIHYDROXYBENZENESULPHONIC ACID IN THE PRODUCTION OF MEDICAMENTS FOR THE TREATMENT OF ANGIODEPENDENT DISEASES SUCH AS CANCER AND PSORIASIS

(57) The invention relates to the use of 2,5-dihydroxybenzenesulfonic acid in the production of medicaments for the treatment of angiodependent diseases. More specifically, the invention relates to the use of the aforesaid compound and, in particular, the calcium and potassium salts thereof, for the treatment of two angiodependent diseases, which present a reduction in the apoptosis, namely cancer and psoriasis. The invention also discloses the antiproliferative, antimigratory, antangiogenic and proapoptotic capacity of said family of com-

pounds in non-quiescent cells. In addition, the invention details the potentiating effect of said compounds on known cytostatic medicines in the treatment of tumours and, specifically, on gliomas. The invention further relates to the therapeutic efficacy of said compounds, based on the combined antiproliferative, antiangiogenic and proapoptotic capacities thereof, in the treatment of chronic psoriatic plaques.

Description**Scope of the Invention**

5 [0001] This invention refers to a pharmaceutical composition that includes the 2,5-dihydroxybenzenesulfonic acid, and its employment in the preparation of a medicine for treatment of diseases characterized by an intense cell proliferation, vascularization (angiodependent diseases) and more specifically angiodependent diseases also having reduction of the apoptosis, as it is the case for example in cancer or psoriasis.

Background of the Invention

10 [0002] Malignant tumors are characterized, besides from the uncontrolled cellular proliferation, by their capacity to invade normal peritumoral tissues. Tumor invasion is a complex process developed according to the following consecutive stages: a) adhesion of the tumor cells to proteins of the extra-cellular matrix; b) degradation of the proteins of the extra-cellular matrix by proteases that create extra-cellular spaces that the tumor cells use to, c) migrate through a dynamic and complex mechanism that requires synthesis of new portions of the cytoplasmic membrane and reorganization of the cytoskeleton (Giese A, Westphal M. Neurosurgery 1996; 39: 235-252). The cells that form the tumor mass invade the normal peritumoral-tissue have their genetic program of cellular death disabled and therefore, the tumor cells that migrate to invade the peritumoral intact tissues, elude the apoptosis (Mariani I et al. Clin Cancer Res 7:2480-2489, 2001). When the grouped tumor cells reach 2 to 3 mm³ volume, the tumor cells synthesize large amounts of angiogenic factors to counteract the hypoxic situation of this primary tumor, (Folkman J. N. Engl J Med 285: 1182-1186, 1971; Carmeliet P, Jain RK. Nature 407: 249-257, 2000; Yancopoulos GD et al. Nature 407: 242-248, 2000) that activate the peritumoral blood vessels so that they form new blood vessels (angiogenesis) that invade the tumor to supply the oxygen and the nutrients and eliminate products from the tumor catabolism. The same cellular processes that occur during the tumor invasion (motility and absence of apoptosis) occur centripetally during tumor angiogenesis. Therefore, the inhibition of the invasive capacity of the tumor cells and of the endothelial cells should produce a delay in tumor growth by inhibiting the tumor expansion, reducing angiogenesis and promoting apoptosis. Therefore, an effective treatment against cancer should inhibit the migration, the angiogenesis and increase apoptosis without producing these effects in normal cells.

25 [0003] There are numerous anti-tumor and antiangiogenic agents at various stages of clinical development in oncology (Brem S. Cancer Control 6: 436-458, 1999), of which a significant number are peptides that the body uses to counteract the effect of the positive regulators of angiogenesis (Hagerdorn M, Bikfalvi A. Crit Rev Onc Hemat 34: 89-110, 2000). However, when these peptides are compared with compounds with a significantly lower molecular weight, their pharmacological inconveniences become evident. On the other hand, it has been proven that different synthetic compounds containing aromatic rings in their molecular structure and acting as inhibitors of the mitogenic activity induced by growth 30 factors are cytotoxic for quiescent or non tumor cells (Lozano RM J Mol Biol 281: 899-9115, 1998). Therefore, there is still need to find compounds with anti-tumor, antiangiogenic and proapoptotic activity with low toxicity for intact, quiescent, non tumor cells. There is presently a great interest for the search of new therapeutic indications for old medicines. In this connection, it has been recently proven that different antibiotics, besides from their antimicrobial activity, have antiproliferative effects, such in the case of rapamycin (Morice MC et al. N Engl J Med 346: 1773-1780, 2002), or of the neomycin (Cuevas P. et al. Neurol Res 224: 389-391, 2002); or are useful as anxiolytics such as norfloxacin (fluoroquinolone) (Johnstone TB et al. Nat Med 10; 31-32, 2004).

35 [0004] Psoriasis is an angiodependent chronic disease that affects 2-3% of the world population and is characterized by epidermic hyperplasia, dermo-epidermic infiltration of inflammatory cells and T lymphocytes, and a very evident development of vascularization, together with a reduction of the cell death due to apoptosis (Kocak M et al. Int J Dermatol 42: 789-793, 2003). Presently, there is no curative treatment for psoriasis. The antipsoriatic treatment may be topical or systemic, depending on the extension and severity of the disease. The mostly used anti psoriatic topical therapy consists of different types of corticoids, but the extended use of these compounds is associated with skin atrophy, stretch marks and telangiectasia (Baker BS, Fry L. Cutis 1999; 64: 315-318). The systemic therapy with immunosuppressant medicines is associated to very severe side effects (Wolina V. et al. Clin Rheumatol 2001; 20: 406-410). For example, the use of cyclosporine for treatment of psoriasis may produce nephrotoxicity (interstitial fibrosis and tubular atrophy), hypertension, hypomagnesaemia, hypercalcemia and hepatic dysfunction (Travis L, Weinberg JM. Drugs of Today 2002; 38: 847-865). The standing use of another immunosuppressant medicine for treatment of psoriasis, tacrolimus, may produce hypertension, nephrotoxicity and immunosuppression (Jegasothy BV et al. Arch Dermatol 1992; 128: 781-785). It has been recently described that the topic application of the tacrolimus immunosuppressant accelerates carcinogenesis 50 in mouse skin (Niwa Y, Terashima T, Sumi H. B J Dermatol 2003; 149: 960-967). Therefore, there is need for new antipsoriatic compounds proving to be efficient without producing evident side effects such as those associated with the most common anti-psoriatic compounds.

55 [0005] The 2,5-dihydroxybenzenesulfonic acid is a derivative of the 2,5-dihydroxybenzoic acid, pharmacologically

prescribed in the form of different salts (mainly calcium, potassium, and magnesium), which provides stability. The 2,5-dihydroxybenzenesulfonic acid has been used since the 70's as an oral vasculotrophic medicine.

[0006] The 2,5-dihydroxybenzenesulfonic acid inhibits platelet aggregation, increase of capilar permeability and blood viscosity in patients with diabetic retinopathy (Bayer J. et al. Dtsch. Mod Wschr 1980; 46: 160-1608; Banaroch I.S. et al. Ophthalmic Res 1985; 17: 131-138; Michal M, Giessinger N. Thromb Res 1988; 51: 593-605). The metabolism and the pharmacokinetics of this compound in the human being is known since year 1974 (Benakis A. et al. Thérapie 1974; 29: 211-219). Recent experiments have proven that the 2,5-dihydroxybenzenesulfonic acid increases the activity of the endothelial isoform of the nitric oxide synthase [endothelial nitric oxyde synthase (eNOS)] in rat endothelial cells without producing cytotoxic effects (Suscheck C. et al. Bt J Pharmacol 1997; 122: 1502-1508). In addition, the 2,5-dihydroxybenzenesulfonic acid potentiates the in vitro relaxation of human penile arteries (Angulo J et al. Br J Pharmacol 2003; 139: 854-862). There is experimental evidence that the 2,5-dihydroxybenzenesulfonic acid (formulated as a calcium or magnesium salts) possesses in vitro antioxidant activities (Brunet J et al. Fundam Clin Pharmacol 12: 205-212, 1998).

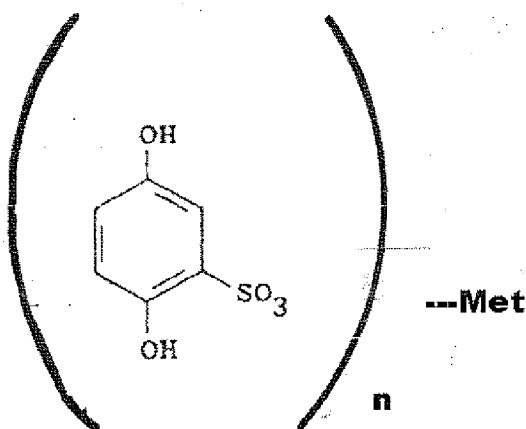
[0007] The present invention is based on the discovery of new activities of the 2,5-dihydroxybenzenesulfonic acid and /or its salts, associated to their antiproliferative, anti migratory, antiangiogenic and proapoptotic capacity in non quiescent cells, activities that combined, justify their employment as a useful compound for treatment of angiodependent diseases such as the case of cancer, characterized by hyperproliferation, cell invasion and excessive angiogenesis, together with a deficit in cell death due to apoptosis, without causing toxicity for non-tumor intact or quiescent cells. Gliomic tumor cells have been used in experiments because gliomas are very invasive tumors with a significant angiogenic capacity and a significant apoptotic deficit (Merzak A, Pilkington GJ. Cancer Metastasis Rev 16:155-177,1997).

[0008] The present invention is also based on the proven fact that the 2,5-dihydroxybenzenesulfonic acid and /or its salts possess, in a combined form, antiproliferative, antiangiogenic, and proapoptotic effects and therefore its therapeutic efficacy has been evaluated in chronic psoriatic plaques characterized by epidermic hyperproliferation, acute dermal angiogenesis and apoptotic deficit (Karasek MA, Cutis 64: 319-322, 1999).

[0009] This invention relates then to the search of new treatments for cancer and other angiodependent diseases an it is based on the fact that the 2,5-dihydroxybenzene sulfonic acid and/ or its salts have proven their capacity to inhibit growth and migration and induce the apoptosis in in vitro tumor cells as well as the capacity to inhibit the in vivo angiogenesis induced by fibroblast growth factor (FGF). Therefore, due to the combination of these abilities, the mentioned compounds become useful for the treatment of malignant tumors and hematological neoplastic diseases as well as for treatment of other severe vascularization related pathologies (angiodependent diseases).

Description of the invention

[0010] The 2,5-dihydroxybenzenesulfonic acid formulated in the form of salt is a commercial product (for example, the potassium salt may be acquired at Merck Farma y Química SA, Mollet del Vallés, Barcelona) with the following molecular formula:



55 in which Met = Metal and n is a function of the metal valence used in the salt. Generally n 0 1 or 2 for being the metal cation former of the salt, univalent (K) or divalent (Ca ó Mg).

[0011] The new biological activities of the 2,5 dihydraxybenzenesulfonic acid do not depend of the cation bond to the benzene ring because the 2,5-dihydroxybenzenesulfonic acid formulated with any salt has similar effects in the inhibition

of cell proliferation, migration and angiogenesis. This invention only describes the activities of the 2,5-dihydroxybenzenesulfonic acid formulated as potassium and calcium salt without forgetting that within the scope of this invention is any pharmaceutically acceptable salt of the compound. The term "pharmaceutically acceptable salts" include metal salts or addition salts which can be used in pharmaceutical forms. The pharmaceutically acceptable salts of the 2,5-dihydroxybenzenesulfonic acid can be obtained from organic or inorganic acids or bases, through conventional methods, by making the appropriate acid or base react with the compound.

[0012] The pharmaceutical compositions containing the 2,5-dihydroxybenzenesulfonic acid can be presented in any adequate administration form, for example, systemic, oral, parenteral, urethral, rectal or topical administration, for which the necessary pharmaceutically acceptable excipients will be included for formulation of the desired form of administration.

[0013] The following examples illustrate and support the invention and should not be considered as limitations of the scope of the invention.

Example 1: Illustrative assay of the anti-proliferative ability of the 2,5-dihydroxybenzenesulfonic acid.

[0014] This in vitro study, was carried out in three different triplicate experiments with rat gliomic cells (C6 line). The cells were cultured in a medium formed by DMEM Dulbecco's modified Eagle's Medium (Gibco. Paisley UK), 7,5% of fetal serum (Gibco) 10 units/ml of penicillin (Gibco) and 10 μ g/ml of streptomycin (Gibco). The cultures were kept in a humid atmosphere at 37°C. To evaluate the effect of the 2,5-dihydroxybenzenesulfonic acid on the cell proliferation, 2 x 10⁴ C6 cells per well were seeded in 24-well (15mm of diameter) plates. The experimental cultures were treated during 48 hours with different micro molar concentrations (μ M) of the compound (calcium or potassium salt of the 2,5-dihydroxybenzenesulfonic acid). The controlled cultures lived 48 hours, without adding the compound. Photographs of the cultures were taken after 48 hours using an inverted microscope and then, the cultures were colored with crystal violet (Merck Farma y Quimica SA. Mollet del Vallés, Barcelona) and processed to determine the number of cells per well, using a spectrum photometric method. As shown in figure 1, treatment with different concentrations of the compound produces a dose-dependent inhibition of cell proliferation, obtaining 88% inhibition with a concentration of 100 μ M of the calcium salt of the 2,5-dihydroxybenzenesulfonic acid (A). With the same concentration of the potassium salt of the 2,5-dihydroxybenzenesulfonic acid, a 74% inhibition was obtained (B). The IC₅₀ is near to 25 μ M for the calcium salt and between 40 and 50 μ M for the potassium salt. Comparing figure 1A with figure 1B, it is observed that to obtain the same percentage of inhibition in cell proliferation after treatment with the calcium salt of the compound, a double concentration of potassium salt is necessary to obtain the same effect. This is due to the fact that the calcium salt of the compound contains two active principle moles (2,5-dihydroxybenzenesulfonic acid) that separate from salt in aqueous solution. Figure 2 shows the image of the C6 cells culture after 48 hours without treatment (A), another image corresponding to the C6 cells culture treated for 48 hours with a concentration of 50 μ M of the calcium salt of the 2,5-dihydroxybenzenesulfonic acid (B) and a third one corresponding to a culture of C6 cells treated during 48 hours with 100 μ M of the potassium salt of the acid (C). This study shows that the treatment with the compound inhibits proliferation in neoplastic cells and corroborates the antiproliferative effect of the compound observed in normal vascular smooth muscular cells stimulated in vitro with mitogenic factors (Parés-Herbute N et al. Int J Angiol 8: S5-S10, 1999). To distinguish if the antiproliferative activity of the 2,5-dihydroxybenzenesulfonic acid is mediated by a cytotoxic or a pro-apoptotic effect, we conducted different experiments detailed in the following example:

Example 2: Illustrative assay of the proapoptotic ability of the 2,5 dihydroxybenzenesulfonic acid.

[0015] This assay was carried out with the C6 cells according to the procedure described in example 1. To demonstrate the proapoptotic effect of the analyzed compounds we have used two different methods that detect the intracellular fragmentation of the DNA and the apoptotic nuclei in situ.

Detection of the intracellular fragmentation of the DNA.

[0016] The enzymatic immunoassay methods to quantify the DNA fragments associated to histones may be considered suitable to determine the onset of apoptosis (Aragane Y et al. J Cell Biol 1998; 140: 171-182). This method allows to differentiate death due to necrosis from death due to apoptosis since in necrosis the cytoplasmic membrane is fragmented and the DNA appears in the culture medium, while in apoptosis, the fragmented DNA remains in the interior of the cell because the plasma membrane remains intact (Aragane Y et al. J Cell Biol 140: 171-182, 1998).

[0017] Using the Cell Death Detection ELISA^{plus} kit (Boehringer Mannheim, Mannheim, Germany) in accordance with the manufacturer's instructions, we have determined the fragmentation of DNA in C6 (2 x 10³) cell cultures at 4, 16, 24 and 48 hours. The controlled cultures did not receive any treatment while from 50 to 200 μ M (Figure 3A) of the potassium salt of the 2,5-dihydroxybenzenesulfonic acid were added to the experimental cultures. Experiments were also conducted adding from 25 to 100 μ M of the calcium salt of the 2,5-dihydroxybenzenesulfonic acid (Figure 3B). All experiments

were performed in triplicate in three different experiments.

[0018] Figures 3A and 3B show the following: a) the antiproliferative effect of the 2,5-dihydroxybenzenesulfonic acid is mainly mediated by a proapoptotic activity; b) the cation bonded to the molecule does not determine the activity of the compound because the proapoptotic effect is similar using the calcium or potassium salt of the compound; c) the highest proapoptotic effect is obtained in cells treated with the compound during 48 hours; d) the maximum effect is obtained with a concentration of 25 μ M for the calcium salt and 50 μ M for the potassium salt, identical to the IC₅₀ in cellular proliferation studies. Once it is proven that the antiproliferative mechanism of the 2,5-dihydroxybenzenesulfonic acid participates in the cell death due to apoptosis, we quantitatively evaluated such effect through a microscopic study of gliomic cells using the following technique:

In situ detection of apoptotic nuclei (TUNEL Technique)

[0019] Three independent experiments were made, repeated three times. The C6 cells from controlled cultures and those from cultures treated during 24 hours with the (50 μ M and 100 μ M of the calcium and potassium salts respectively) were adhered to glass slides and fixed with a 4% paraformaldehyde buffered solution (pH 7,4) for one hour at the laboratory temperature. Afterwards, the cells were washed and permeabilized with a 0.1% solution of Triton X-100. Then the cells were washed before applying the TUNEL technique [(terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick and labeling (Gavrieli Y, Sherman Y, Bensasson SA. J Cell Biol 119: 493-501, 1992). A kit for in situ detection of apoptotic nuclei (In situ Cell Death Detection Kit Boehringer Mannheim, Mannheim, Germany) was used. The different stages of the technique were followed in accordance with the instructions of the kit manufacturer. Finally, the cells were colored with green light (Fluka, AG, Switzerland). The TUNEL reaction only appears in the apoptotic nuclei.

[0020] Although very similar results were obtained with the calcium and potassium salt of the compound, object of the invention, only the results obtained with the potassium salt of the compound are shown. Cells were counted in 6 different fields in twelve slides where the cells from the 6 control cultures and the 6 cultures treated with the 2,5-dihydroxybenzenesulfonic acid (100 μ M) had adhered. The total number of non apoptotic and apoptotic cells was as follows:

C6 Cells	Apoptotic Nuclei	Normal Nuclei
Control Cells	138	5954
Treated Cells	3846	354

The total number of treated cells is lower than the total number of control cells due to the antiproliferative effect of the compound.

[0021] The images of Figure 4 show an area of an experiment of a control culture (A and B) and of another culture treated with the compound (C and D) in which the TUNEL technique was employed. As shown in the images, only two apoptotic nuclei are observed on the control cells while in the treated cells with the compound object of the invention there are 107 apoptotic nuclei and only 8 normal nuclei (non apoptotic).

[0022] These data show that the 2,5-dihydroxybenzenesulfonic acid is a compound with an important proapoptotic activity useful to induce tumor apoptosis. Given that it has been proven that the 2,5-dihydroxybenzenesulfonic acid inhibits apoptosis in normal human cells (Braber R, Farine JC, Lora GA. Apoptosis 4: 4111-49, 1998), this compound is a strong molecule candidate for treatment of cancer.

[0023] One of the mechanisms involved in the therapeutic failure of chemotherapy and radiotherapy is the inefficacy of these treatments to induce cellular death by apoptosis, mainly due to the hyper expression of antiapoptotic proteins in tumor cells (Sellers WR, Fisher DE. J Clin Invest 104: 1655-1661, 1999; Branch P. et al. Oncogene 19: 3138-3145, 2000). Therefore, the proapoptotic compounds may be of great clinical use as an adjuvant in chemotherapy and radiotherapy treatments.

[0024] Once the proapoptotic effect of the 2,5-dihydroxybenzenesulfonic acid was demonstrated, we evaluated the ability of this compound to increase the antiproliferative effect of the different cytostatic medicines. The following example demonstrates how the 2,5-dihydroxybenzenesulfonic acid is capable of increasing the therapeutic efficacy of the different cytostatic compounds used in oncology such as cisplatin, vincristine, paclitaxel and 5-fluorouracil.

Example 3: Illustrative assay of the ability of the 2,5-dihydroxybenzene sulfonic acid in potentiation of chemotherapy

[0025] We used for this study C6 cells cultured in vitro under the same conditions described in example 1. 1x 10³ cells per well were cultured in 24-well plates. Three types of treatment were made: a) 24 hours after the seeding, the cells were separately treated with each one of the following medicines; cisplatin (5 μ g/ml), vincristine (0,1 μ g/ml), paclitaxel

(5 μ g/ml) and 5-fluorouracil (100 μ g/ml); b) 24 hours after the seeding, the cells were treated jointly with the 2,5-dihydroxybenzenesulfonic acid (potassium salt, 100 μ M) and with each one of the following medicines; cisplatin (5 μ g/ml) vincristine (0,1 μ g/ml), paclitaxel (5 μ g/ml) and 5-fluorouracil (100 μ g/ml); c) at the time of the seeding (Day 0), the cells were pre-treated with the 2,5-dihydroxybenzenesulfonic acid (potassium salt, 100 μ M). Next day the cultures were treated also with each one of the following medicines: cisplatin (5 μ g/ml) vincristine (0,1 μ g/ml), paclitaxel (5 μ g/ml) and 5-fluorouracil (100 μ g/ml). The controlled cultures did not receive treatment for 2 days. After 48 hours (day 2), the cells of identical shape to the ones used in example 1 were evaluated in all the cultures. This study was carried out in triplicate independent experiments repeated three times.

[0022] Figure 5 (A, B, C and D) shows the histograms of the experiments performed to evaluate the effect of the 2,5-dihydroxybenzenesulfonic acid in the potentiation of the different cytostatic medicines. Treatment with cisplatin, vincristine and 5-fluorouracil produces an inhibition of 50% in proliferation of C6 cells, while the treatment with paclitaxel obtains 67% of inhibition of the cellular proliferation. The combined treatment of the 2,5-dihydroxybenzenesulfonic acid + the cytostatic medicines (cisplatin, vincristine and 5-fluorouracil) produces an inhibition of 84% in cellular proliferation. The combined treatment with 2,5-dihydroxybenzenesulfonic acid + paclitaxel produces 86% in the inhibition of the cellular proliferation. When cellular cultures are pre-treated with the 2,5-dihydroxybenzenesulfonic acid and afterwards with the following cytostatic medicines: cisplatin, vincristine and 5-fluorouracil, an inhibition of 90% is obtained in the cell proliferation. When paclitaxel is used, the inhibition in cellular proliferation reaches up to 92%.

[0023] The above mentioned results demonstrate that the simultaneous treatment of the 2,5-dihydroxybenzenesulfonic acid with the chemical therapy agents, increases their therapeutic efficacy and besides this chemical potentiation effect is higher when the cells has been pre-treated with the 2,5-dihydroxybenzenesulfonic acid. These data support the use of the 2,5-dihydroxybenzenesulfonic acid as an adjuvant in the treatment associated with chemical therapy and radiotherapy.

Example 4: Illustrative assay of the antimigration ability of the 2,5 dihydroxybenzenesulfonic acid

[0024] This assay was carried out in three different triplicate experiments. To evaluate the ability of the 2,5-dihydroxybenzenesulfonic acid in the inhibition of cellular migration C6 2 x 10⁵ cells cultured in vitro in 20 mm plates were used. A longitudinal lesion was made with a sterile micropipette (day 0) to the control cultures and in cultures treated with 100 μ M of the potassium salt of the 2,5-dihydroxybenzenesulfonic acid. Digital photos were taken using a photographic system connected to a luminous microscope and the area of the lesion was delimited using a computerized morphometric program (Moticam. Motic. Barcelona). Photographs were taken again after 24 hours, and the borders of the lesion were marked overlapping the first two photos (day 0) with those obtained after 24 hours to calculate the percentage of the injured area covered by the migratory cells. These values were represented as a percentage of the regeneration obtained with the treatment. Figure 6 shows a typical example of a control experiment (A) and another experiment in which the cells were treated during 24 hours with the compound object of the invention (B). As observed in this Figure, the non treated cells completely regenerate the lesion (Figure 6A) while the cells treated with the compound are not capable of migrating and cover all the area of the lesion (Figure 6B). Figure 7 that represents the percentage data of all the experiments shows that the 2,5-dihydroxybenzenesulfonic acid inhibits up to 64% of migration of tumor cells.

Example 5: Illustrative Assay of the antiangiogenic ability of the 2,5-dihydroxybenzenesulfonic acid

[0025] We used for this assay the chorioallantoic membrane of a chick embryo for testing the activity of antiangiogenic substances in vivo (Zilberberg L. et al. J Biol Chem 2003; 278: 35564-35573). We used a proangiogenic compound, the basic form of the fibroblast growth factor (bFGF) (Meghna U et al. Blood 2003; 102: 2108-2114).

[0026] Fertilized eggs are kept in a incubator at 37°C with a humidity of 80%. After 4 days, a hole is made in the narrowest end of the egg shell to collect 1ml of albumin Then, the hole is covered with a paraffin film (Parafilm M Laboratory Film Chicago IL. USA). This procedure allows creating an air chamber that prevents the embryo to adhere to the upper part of the shell. On day 13 of incubation, the shell is split at the air chamber level to perform the treatment. Twenty embryos are treated with 5 μ l of a solution of 3 μ g of bFGF + 0.1% heparin, soaked in a nitrocellulose paper disc. Afterwards the shell is sealed with a paraffin film. Next day, in half of the embryos (n =10) the shell is uncovered to soak again the nitrocellulose paper disc with 100 μ M of potassium salt of the 2,5-dihydroxybenzenesulfonic acid dissolved in physiological saline (5 μ l). The hole in the shell is then covered again with a paraffin film. On day seventeen the experiment ends, taking photographs of the nitrocellulose piece for the comparison study.

[0027] Figure 8 presents two images corresponding to an embryo treated with 3 μ g of bFGF + 0, 1% heparin (A) and another embryo to which 100 μ M of a potassium salt solution of the 2, 5-dihydroxybenzenesulfonic acid was added on the next day (B) Image A shows how the nitrocellulose disc is invaded by blood vessels while Image B shows a very scarce vascular invasion in the disc. The morphometric quantification of the images of the nitrocellulose discs using a computerized system (Moticam Motic. Barcelona) shows the antiangiogenic effect of the compound (area of the disc

covered by blood vessels in embryos treated with bFGF + heparin = $35 \pm 8.6\%$ vs. area of the disc covered by blood vessels in embryos treated with bFGF + heparin + potassium salt of the 2,5-dihydroxybenzene sulfonic acid = $2 \pm 1.5\%$; p<0,0001; unpaired student's t-test). Similar effects were obtained using 50 μM of the calcium salt of the compound. This experiment shows that the compound object of this invention has an antiangiogenic activity for being capable of neutralizing the angiogenic effect induced by bFGF.

Example 6: Assay on psoriatic lesions

[0028] We used for this study the potassium salt of the 2,5-dihydroxybenzenesulfonic acid formulated at 2,5 and 5% in cream for being this type of formulation a usual procedure for topical treatment of skin diseases. The selected concentrations of the salts of the 2,5-dihydroxybenzenesulfonic acid are within the range of the concentrations used for treatment of diabetic retinopathies: 6 tablets per day of 500mg of calcium salt of the 2,5-dihydroxybenzenesulfonic acid (Benakis A et al Thérapie 1974; 29: 211-219). As aqueous phase of the cream we have used distilled water. The fatty phase can be constituted by cetyllic alcohol, stearic alcohol or vaseline. The span is an efficient emulsifier in the preparation of the cream. Although both formulations (2,5 and 5%) of the product show to be clinically efficient, the best therapeutic benefit is obtained with the concentration at 5%. Therefore, we present the results obtained with the acid formulated in the cream at 5%. The following example illustrates the formulation of an efficient cream for the topic treatment of psoriasis, by way of example and not of limitation of the scope of the invention.

I.- Active Part (potassium salt of the 2,5-dihydroxybenzenesulfonic acid at 5,6%)

II.- Inactive Part (as excipients cetyllic alcohol (2,5%), stearyl alcohol (2,5%), liquid vaseline (30%), white soft paraffin (20%), sorbitan oleate (5%) and distilled water (c.s.p. 100g).

[0029] The clinical efficacy of the treatment was evaluated according to the index that quantifies the desquamation signs (D), erythema (E) and infiltration (I) to which the following assessment was assigned: (0) absent; (1) slight; (2) moderate and (3) severe (Freeman AK et al. J Am. Acad Dermat 2003; 48: 564-568). Figure 9 shows three images: before treatment, six and thirteen days after treatment of the same chronic psoriatic plaque located in the extension area of the left elbow treated with the potassium salt of the 2,5-dihydroxybenzenesulfonic acid at 5%. As can be observed, the topical treatment two times at day with a cream containing the potassium salt of the 2,5-dihydroxybenzene sulfonic acid produces an early (6 days) very notable "clearance" of the plaque with almost total disappearance of hyperkeratosis. The therapeutic efficacy of the cream is more evident at the end of the second week of treatment. The treatment produces a significant reduction of the global values of the DEI index (DEI global pre-treatment = $6 \pm 1,57$ vs. DEI global post-treatment = $1 \pm 0,58$; p<0,0001; unpaired student's t-test).

Figures Captions

[0030]

1. Histogram showing the antiproliferative effect of the treatment with different concentrations of the (A) calcium and (B) potassium salts of the 2,5-dihydroxybenzenesulfonic acid in cultures of C6 cells after 48 hours of treatment. Ordinates: Absorbance at 595 nm; Abscises: concentration: μM .

2. Panel A shows the aspect of a control culture of C6 cells after 48 hours. Panel B shows an image of a culture of C6 cells treated during 48 hours with 50 μM of the 2,5-dihydroxybenzenesulfonic acid (calcium salt). Panel C shows a culture of C6 treated during 48 hours with 100 μM of the potassium salt of the 2,5-dihydroxybenzenesulfonic acid.

3. Representative histograms in which it is observed that the antiproliferative effect of the 2,5-dihydroxybenzenesulfonic acid is not due to necrosis (white histogram) but to apoptosis (lined histogram). A: treatment with the calcium salt of the 2,5-dihydroxybenzenesulfonic acid. B: Treatment with the potassium salt of the 2,5-dihydroxybenzenesulfonic acid. Ordinates: Absorbance at 405 nm; Abscises: time in hours.

4. Images of gliomic C6 cells processed with the TUNEL technique for in-situ detection of apoptotic cells . The apoptotic nuclei are shown dark and the nucleus and cytoplasm of the cell of the non apoptotic cells are shown in light color. The arrows indicate apoptotic nucleus. A and B control cells, C and D cells treated with 2,5-dihydroxybenzenesulfonic acid. Photographs B and D correspond to a zoom of the boxes of A and C photographs respectively.

5. Histograms demonstrating the potentiating effect on chemotherapy (assessed as an antiproliferative effect) of

the 2,5-dihydroxybenzenesulfonic acid, with different cytostatic compounds A) Cisplatin (5 µg/ml); B) Vincristine (0.1 µl/ml); C) Paclitaxel (5Ug/ml) and D) 5-fluorouracil (100 µg/ml). Ordinates: Absorbance 595 nm; Abscises: white histogram (control); dotted (cytostatic; day 1); lined histogram (2,5-dihydroxybenzenesulfonic acid + cytostatic; day 1); square histogram (2, 5-dihydroxybenzenesulfonic acid (day 0) + cytostatic; day 1).

5 6. Photographic images of cellular migration in a control experiment and other experiments where the cells were treated with the 2,5-dihydroxybenzenesulfonic acid (B). The control cells totally regenerate one lesion made during the culture, while the cellular migration of the cells treated with the 2,5-dihydroxybenzenesulfonic acid, was unable to fully cover the affected area of the culture. The horizontal lines delimit the initial longitudinal lesion made in the cultures.

10 7. Histogram representing the migratory ability of the C6 cells in controlled cultures (white histogram) and in cultures treated with the 2,5-dihydroxybenzenesulfonic acid (black histogram). The migratory ability is expressed (ordinates) as a percentage of regeneration (percentage of the area covered of a longitudinal lesion made in the cultures)

15 8. Images of two chicken embryos with 17 days of incubation. Panel A corresponds to an embryo treated with 3 µg of bFGF + 0, 1% of heparin. Panel B shows the aspect of an embryo treated with 3 µg of bFGF + 0.1% de heparin + 100 µM of the potassium salt of the 2, 5-dihydroxybenzenesulfonic acid. Panel A shows the antiangiogenic effect of the 2, 5-dihydroxybenzenesulfonic acid because the nitrocellulose disc used as releasing vehicle of the substance appears almost without any vessels.

20 9. Images of a hiperkeratosic psoriatic plaque located in the rear region of the left elbow. Image A represents the aspect of the psoriatic plaque before initiating treatment. Image B is an aspect of the same plaque after six days of treatment with a cream at 5% containing as an active component the potassium salt of the 2,5-dihydroxybenzenesulfonic acid. Image C shows the aspect of the psoriatic plaque after two weeks of treatment with the potassium salt of the 2,5-dihydroxybenzenesulfonic acid formulated at 5%. The numbers shown in the images correspond to the day on which the photographs were taken.

30 Claims

1. Use of the 2,5-dihydroxybenzenesulfonic acid or of any of its pharmaceutically acceptable salts for manufacturing of medicines applicable to the treatment of angiodependent diseases.
- 35 2. Use according to claim 1 in which the angiodependent disease also presents a reduction of apoptosis.
3. Use according to any of claims 1 or 2 in which the manufactured medicine is for application in the treatment of cancer.
- 40 4. Use according to claim 3 **characterized** because the manufactured medicine is used to increase the antiproliferative effect of the cytostatic medicines in the treatment of cancer.
5. Use according to any of claims 1 to 4 **characterized** because the salt preferred for the manufacturing of the medicine is the potassium salt of the 2,5-dihydroxybenzene sulfonic acid.
- 45 6. Use according to any of the claims 1 to 4 **characterized** because the salt preferred for producing the medicine is the calcium salt of the 2,5-dihydroxybenzene sulfonic acid.
7. Use according to any of the above claims 1 to 6 in which the manufactured medicine also includes an adequate amount of at least one pharmaceutically acceptable excipient.
- 50 8. Use according to claim 1 in which the manufactured medicine is for application in the treatment of psoriasis.
9. Use according to claim 8 **characterized** because the salt preferred for production of the medicine is the potassium salt of the 2,5-dihydroxybenzenesulfonic acid.
- 55 10. Use according to claim 8, **characterized** because the salt preferred for manufacturing of the medicine is the calcium salt of the 2,5-dihydroxybenzenesulfonic acid.

11. Use according to any of the above claims 8 to 10 in which the manufactured medicine also includes adequate amount of at least one pharmaceutically acceptable excipient.

5 12. Use according to any of the claims 8 to 11 **characterized** because the medicine consists on a topical application formulation.

13. Use according to claim 12 **characterized** because the medicine is a cream or unguent whose composition includes:

10 • A pharmaceutically efficient amount of the 2,5-dihydroxybenzenesulfonic acid or of any of its pharmaceutically acceptable salts.
• A pharmaceutically acceptable amount of at least one alcohol.
• A pharmaceutically acceptable amount of at least one emulsifier.
• A pharmaceutically acceptable amount of at least one excipient.
• A pharmaceutically acceptable amount of at least one excipient comprising a lipid phase, particularly vaseline
15 • Distilled water

14. Use according to claim 13 **characterized** because the medicine is a cream or unguent that presents a composition that includes:

20 • 5% of the potassium salt of the 2,5-dihydroxybenzenesulfonic acid
• 2,5% of cetyllic alcohol
• 2,5% of stearic alcohol
• 30% of liquid vaseline
• 30% of white soft paraffin
25 • 5% span (sorbitan oleate)
• q.s 100 g of distilled water

30

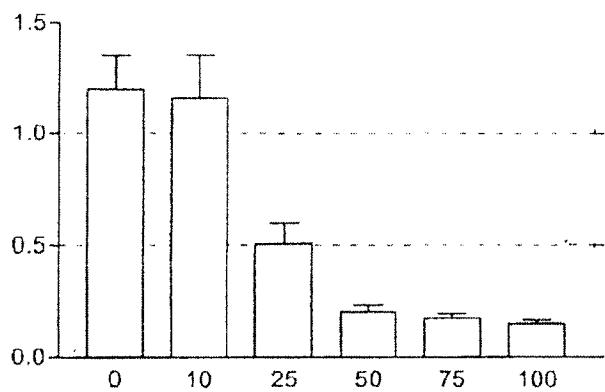
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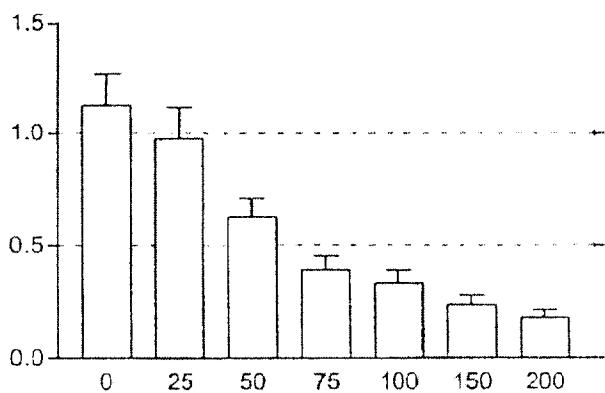
45

50

55



A



B

FIG. 1

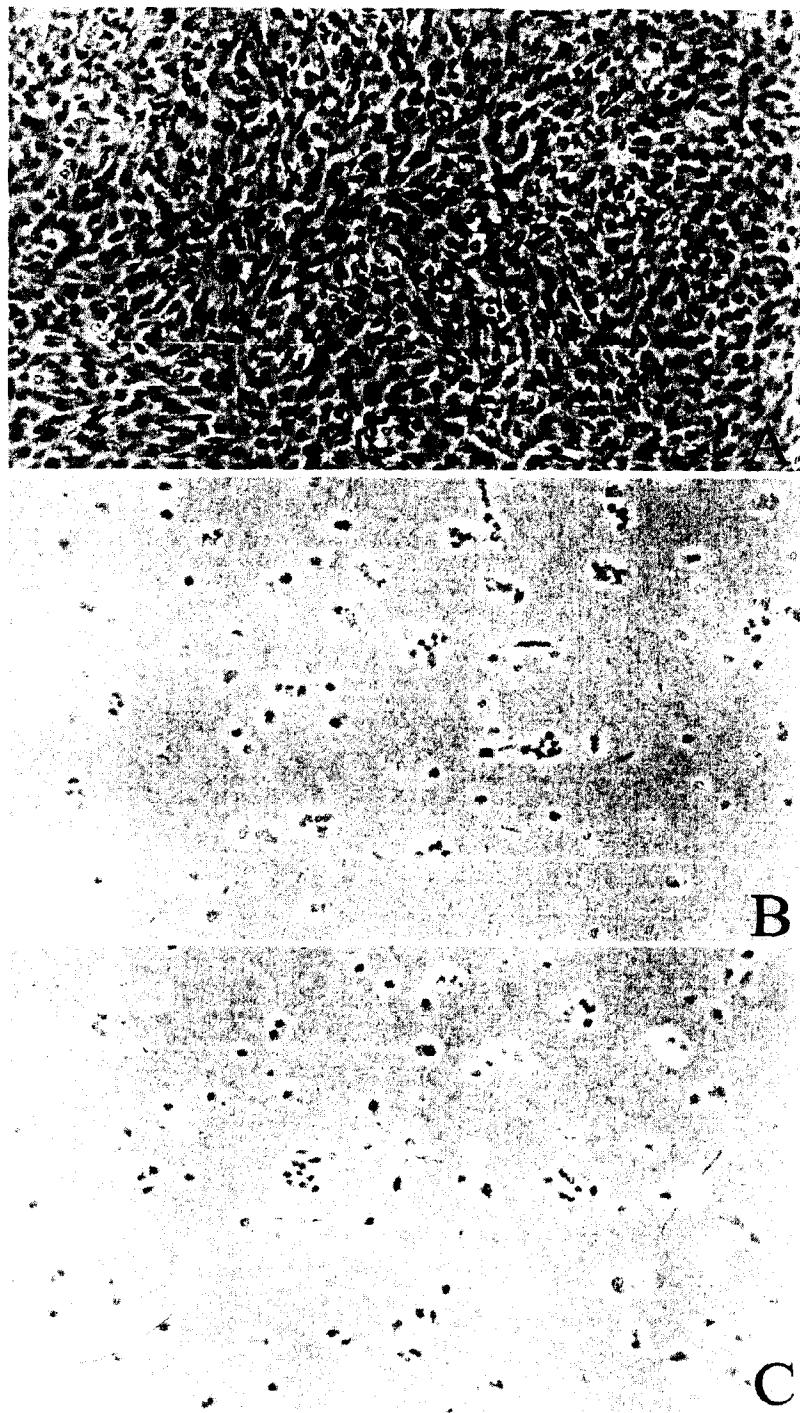
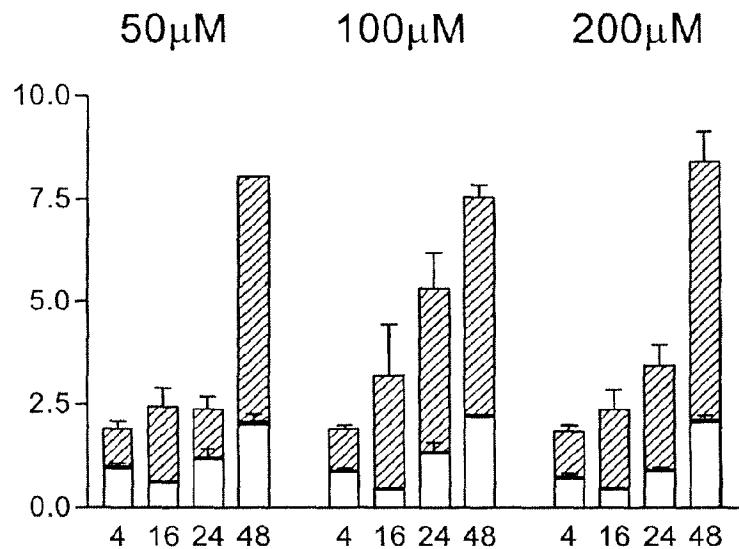
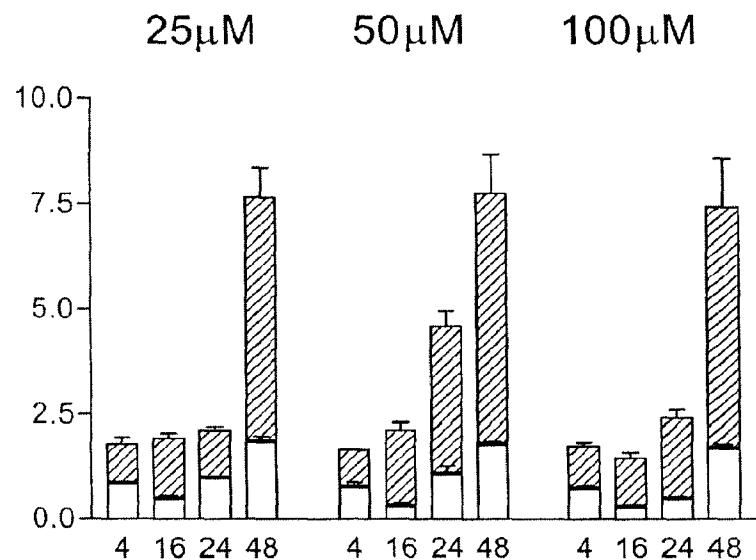


FIG. 2

A**B****FIG. 3**

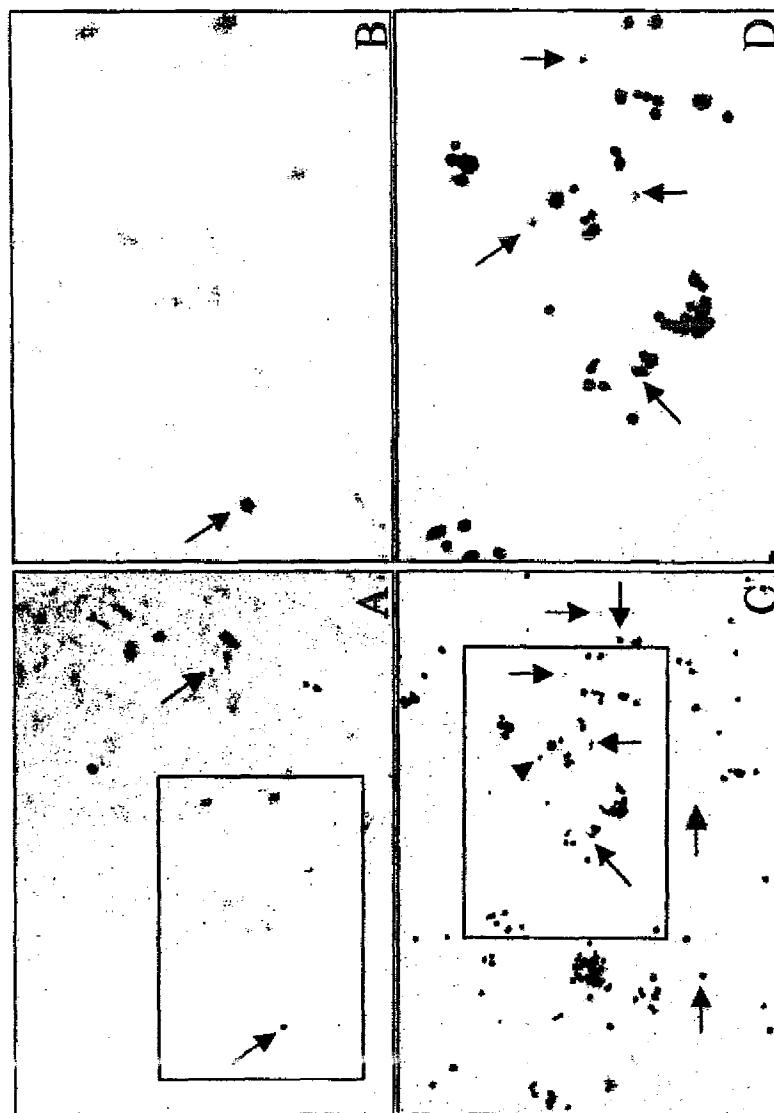
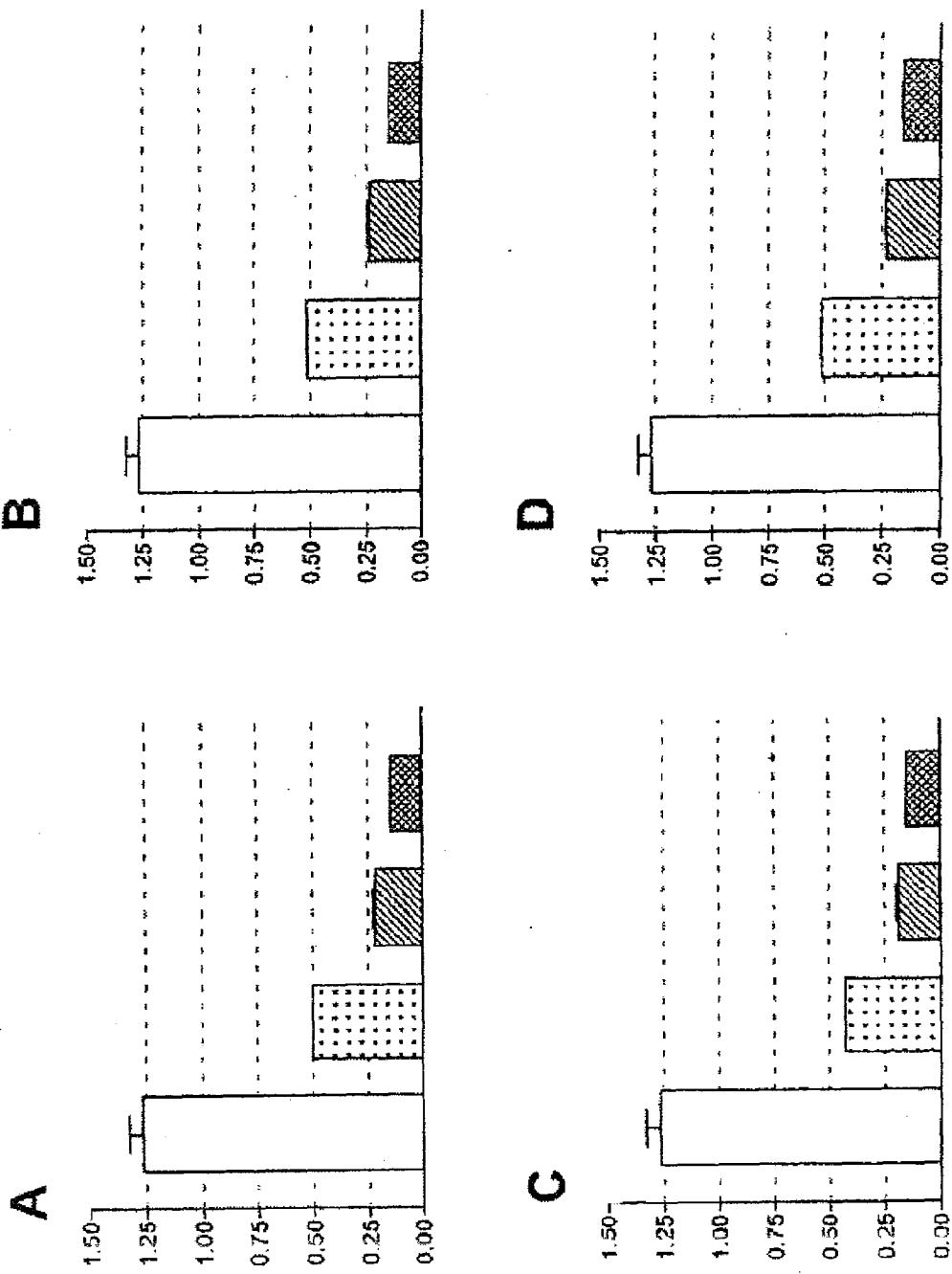


FIG. 4

**FIG. 5**

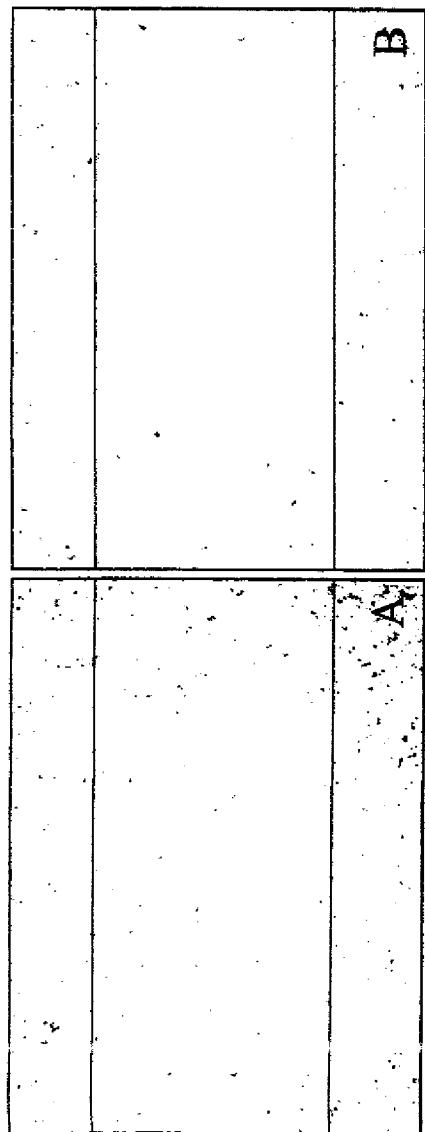


FIG. 6

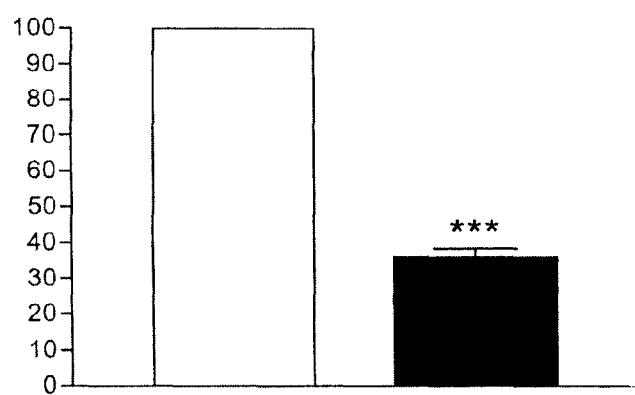


FIG. 7

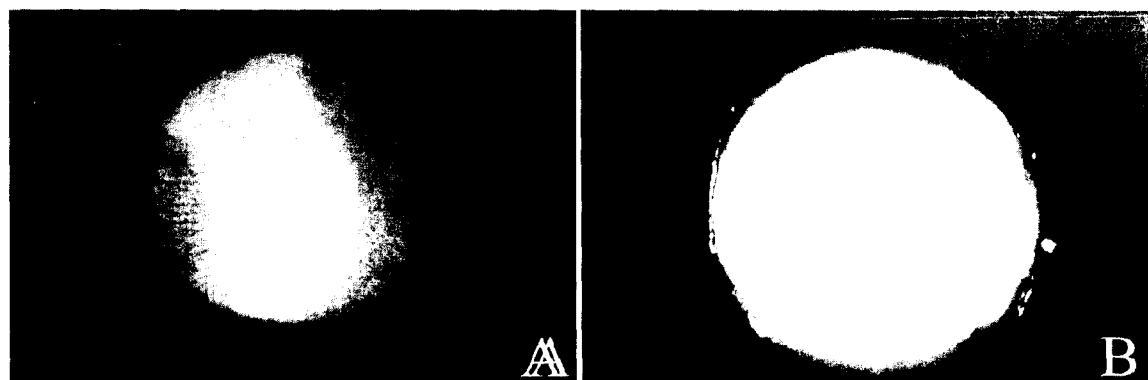


FIG. 8



FIG. 9

INTERNATIONAL SEARCH REPORT

International application No.

PCT/ES 2005/070017

A. CLASSIFICATION OF SUBJECT MATTER		
IPC ⁷ A61K 31/185, , A61P 35/00, A61P 17/06 According to International Patent Classification (IPC) or to both national classification and IPC		
B. FIELDS SEARCHED		
Minimum documentation searched (classification system followed by classification symbols) IPC⁷ A61K		
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched		
Electronic data base consulted during the international search (name of data base and, where practicable, search terms used) CIBEPAT, EPODOC, REGISTRY, HCAPLUS, EMBASE, BIOSIS, MEDLINE.		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	NOUR A.F. & SALAM S. Preliminary clinical study with calcium dobesilate in fibrocystic disease of the breast, a pilot study. <i>Invasive Treatment</i> . 1986, Volume 12, Nº 3, pages 233-242. The whole document.	1,2,5-7
X	RUIZ, E. & TEJERINA, T. Calcium dobesilate increases endothelium-dependent relaxation in endothelium-injured rabbit aorta. <i>Pharmacological Research</i> . 1998, Volume 38, Nº 5, pages 361-366. (abstract) HCAPLUS [on line] [recovered on 02.02.2005]. Recovered from: STN International, Columbus, Ohio (EE.UU.). Access Nr. 1999:6013.	1,6
A	GRABER, R. y col. Calcium dobesilate protects human peripheral blood mononuclear cells from oxidation and apoptosis. <i>Apoptosis</i> . 1998, Volume 3, Nº 1, pages 41-49. (abstract) HCAPLUS [on line] [recovered on 02.02.2005]. Recovered from: STN International, Columbus, Ohio (EE.UU.). Access Nr. 1998:171183.	1,2,6
<input type="checkbox"/> Further documents are listed in the continuation of Box C. <input type="checkbox"/> See patent family annex.		
<p>* Special categories of cited documents: "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier document but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed</p> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art "&" document member of the same patent family</p>		
Date of the actual completion of the international search 13 June 2005 (13.06.05)	Date of mailing of the international search report 22 June 2005 (22.06.05)	
Name and mailing address of the ISA/ SPTO	Authorized officer	
Facsimile No.	Telephone No.	

Form PCT/ISA/210 (second sheet) (July 1992)

REFERENCES CITED IN THE DESCRIPTION

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Non-patent literature cited in the description

- GIESE A ; WESTPHAL M. *Neurosurgery*, 1996, vol. 39, 235-252 [0002]
- MARIANI I et al. *Clin Cancer Res*, 2001, vol. 7, 2480-2489 [0002]
- FOLKMAN J. N. *Engl J Med*, 1971, vol. 285, 1182-1186 [0002]
- CARMELIET P ; JAIN RK. *Nature*, 2000, vol. 407, 249-257 [0002]
- YANCOPOULOS GD et al. *Nature*, 2000, vol. 407, 242-248 [0002]
- BREM S. *Cancer Control*, 1999, vol. 6, 436-458 [0003]
- HAGERDOM M ; BIKFALVIA. *Crit Rev Onc Hemat*, 2000, vol. 34, 89-110 [0003]
- LOZANO RM. *J Mol Biol*, 1998, vol. 281, 899-9115 [0003]
- MORICE MC et al. *N Engl J Med*, 2002, vol. 346, 1773-1780 [0003]
- CUEVAS P et al. *Neurol Res*, 2002, vol. 224, 389-391 [0003]
- JOHNSTONE TB et al. *Nat Med*, 2004, vol. 10, 31-32 [0003]
- KOCAK M et al. *Int J Dermatol*, 2003, vol. 42, 789-793 [0004]
- BAKER BS ; FRY L. *Cutis*, 1999, vol. 64, 315-318 [0004]
- WOLINA V. et al. *Clin Rheumatol*, 2001, vol. 20, 406-410 [0004]
- TRAVIS L ; WEINBERG JM. *Drugs of Today*, 2002, vol. 38, 847-865 [0004]
- JEGASOTHY BV. *Arch Dermatol*, 1992, vol. 128, 781-785 [0004]
- NIWA Y ; TERASHIMA T ; SUMI H. B. *J Dermatol*, 2003, vol. 149, 960-967 [0004]
- BAYER J. et al. *Dtsch. Mod Wschr*, 1980, vol. 46, 160-1608 [0006]
- BANARROCH I.S. et al. *Ophthalmic Res*, 1985, vol. 17, 131-138 [0006]
- MICHAL M ; GIESSINGER N. *Thromb Res*, 1988, vol. 51, 593-605 [0006]
- BENAKIS A. et al. *Therapie*, 1974, vol. 29, 211-219 [0006]
- SUSCHECK C. et al. *Bt J Pharmacol*, 1997, vol. 122, 1502-1508 [0006]
- ANGULO J et al. *Br J Pharmacol*, 2003, vol. 139, 854-862 [0006]
- BRUNET J et al. *Fundam Clin Pharmacol*, 1998, vol. 12, 205-212 [0006]
- MERZAK A ; PILKINGTON GJ. *Cancer Metastasis Rev*, 1997, vol. 16, 155-177 [0007]
- KARASEK MA. *Cutis*, 1999, vol. 64, 319-322 [0008]
- PARÉS-HERBUTE N et al. *Int J Angiol*, 1999, vol. 8, S5-S10 [0014]
- ARAGANE Y et al. *J Cell Biol*, 1998, vol. 140, 171-182 [0016] [0016]
- GAVRIELI Y ; SHERMAN Y ; BENSASSON SA. *J Cell Biol*, 1992, vol. 119, 493-501 [0019]
- BRABER R ; FARINE JC ; LORA GA. *Apoptosis*, 1998, vol. 4, 4111-49 [0020]
- SELLERS WR ; FISHER DE. *J Clin Invest*, 1999, vol. 104, 1655-1661 [0020]
- BRANCH P. et al. *Oncogene*, 2000, vol. 19, 3138-3145 [0020]
- ZILBERBERG L. et al. *J Biol Chem*, 2003, vol. 278, 35564-35573 [0025]
- MEGHNA U et al. *Blood*, 2003, vol. 102, 2108-2114 [0025]
- BENAKIS A et al. *Therapie*, 1974, vol. 29, 211-219 [0028]
- FREEMAN AK et al. *J Am. Acad Dermat*, 2003, vol. 48, 564-568 [0029]